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Nitric Oxide and Cytochrome *c* Oxidase: Mechanisms of Inhibition and NO Degradation

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NO inhibits mitochondrial respiration by reacting with either the reduced or the oxidized binuclear site of cytochrome *c* oxidase, leading respectively to accumulation of cytochrome a_3^{2+}-NO or cytochrome a_3^{3+}-NO_2^- species. Exploiting the unique light sensitivity of the cytochrome a_3^{2+}-NO, we show that under turnover conditions, depending on the cytochrome c^{2+} concentration, either the cytochrome a_3^{2+}-NO or the nitrite-bound enzyme is formed. The predominance of one of the two inhibitory pathways depends on the occupancy of the turnover intermediates. In the dark, the respiration recovers at the rate of NO dissociation ($k' = 0.01\ s^{-1}$ at 37°C). Illumination of the sample speeds up recovery rate only at higher reductant concentrations, indicating that the inhibited species is cytochrome a_3^{2+}-NO. When the reaction occurs with the oxidized binuclear site, light has no effect and NO is oxidized to harmless nitrite eventually released in the bulk, accounting for catalytic NO degradation.

Key Words: **nitric oxide; cytochrome oxidase; inhibition mechanism; cell metabolism; NO degradation.**

Over the past 5 years, evidence from kinetic experiments and computer simulations (1–3) led to some understanding of the mechanism of inhibition of cytochrome *c* oxidase by NO, a phenomenon of great pathophysiological significance (4–8), given that NO is produced also in mitochondria (9, 10). Consistently with the rapid reaction of NO with reduced cytochrome a_3 ($k_{on} = 0.4–1 \times 10^8\ M^{-1}\ s^{-1}$, (11)), accumulation of the reduced NO adduct was observed (1, 3). This state of the enzyme is obviously inactive, but as NO is removed from the medium, the enzyme reverts fairly rapidly (seconds to minutes) to the fully active turnover species (1, 3), with spectral changes corresponding to the dissociation of NO from reduced cytochrome a_3^{2+}. Later, it was found that NO also reacts (in a 1:1 stoichiometry) with the oxidised binuclear site once deprived of chloride, with a $k_{on} = 5 \times 10^5\ M^{-1}\ s^{-1}$ (12, 13). Rapid reduction of cytochrome a/Cu_A *via* reaction of NO with Cu_B^{2+} (in the oxidized enzyme) is associated to formation of nitrite in the active site pocket of the enzyme, and (reversible) inhibition (2). The reactivity of the $\mathbf{P_M}$ and **F** intermediates, statically generated, was found similar in stoichiometry and kinetics to that of the fully oxidized enzyme, with the generation of bound nitrite (unpublished data).

We are therefore faced with two different inhibition mechanisms, which may both be significant in the cell depending on the population of the relevant species and their intrinsic reactivity with NO. In this study we show that it is possible to reveal which of the two mechanisms prevails under a given set of experimental conditions, by simply evaluating the sensitivity to light of the steady-state respiration rate of cytochrome *c* oxidase in the presence of NO and O_2. The results seem to provide a rational synthesis of the inhibition of respiration by NO and show that cytochrome c oxidase is the respiratory complex primarily involved in the short-term response to NO.

MATERIALS AND METHODS

Materials. Horse heart cytochrome *c*, ascorbate, ruthenium(III) hexamine, sodium nitrite and tetramethyl-*p*-phenylenediamine (TMPD) were from Sigma (St. Louis, MO); dodecyl-β-D-maltoside from Biomol (Hamburg, Germany). Stock solutions of NO (Air Liquide, Paris, France) were prepared equilibrating degassed water with the pure gas (purged from higher level oxides) at 1 atm and at 20°C ([NO] = 2.1 ± 0.1 mM). Experiments were carried out in 0.1 M K^+/HEPES, pH 7.3, +0.1% dodecyl-β-D-maltoside and at 20°C (unless otherwise stated).

Cytochrome *c* oxidase, purified from beef heart (14), is expressed as functional unit (aa_3) ($\Delta\epsilon_{444\ (red\text{-}ox)} = 156\ mM^{-1}\ cm^{-1}$). Human hemoglobin (Hb), purified according to (15), is expressed on the heme basis, ($\epsilon_{555} = 12.5\ mM^{-1}\ cm^{-1}$ in the deoxygenated state). The concentration

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of cytochrome *c* was determined using $\Delta\epsilon_{550\,(red\text{-}ox)} = 18.6\ mM^{-1}\ cm^{-1}$. Static spectra were recorded with a double-beam spectrophotometer (Jasco V-570) with a light path of 1 cm. All concentrations are after mixing, unless otherwise stated.

Time-resolved spectroscopy. Stopped-flow experiments were carried out with a rapid mixing instrument equipped with a diode-array (DX.17MV, Applied Photophysics, Leatherhead, UK). The instrument uses a 150 W Xenon lamp, has a light path of 1 cm and can acquire up to 500 spectra with an acquisition time of 2.5 ms per spectrum. Curve fitting was performed with the software MATLAB (MathWorks) and analysis of time-resolved spectra with the singular value decomposition (SVD) algorithm (16).

O_2 consumption. Oxygen consumption catalyzed by cytochrome oxidase has been measured polarographically with a Clark-type O_2-electrode (Ysi Model 5300, Yellow Springs Instruments Co., Ohio) interfaced with a chart recorder. In a typical experiment, cytochrome oxidase was added to air-equilibrated buffer containing ascorbate, TMPD and cytochrome *c*. Addition of NO inhibits the enzyme, and inhibition can be removed by addition of HbO_2 (in excess over NO). When necessary, measurements have been carried out focussing on the reaction chamber the light (heat filtered) of a 150 W tungsten lamp. The observed traces were digitized and analyzed with the software MATLAB.

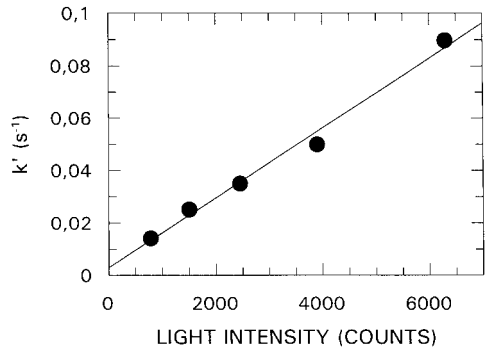


FIG. 1. Rate of NO dissociation from reduced oxidase: the effect of light. Cytochrome *c* oxidase (4 μM) was reduced by anaerobic incubation with 10 mM ascorbate for 2 h. Afterward, 20 μM NO was added and the resulting nitrosyl adduct was mixed in the diode-array stopped-flow against air-equilibrated buffer at 20°C, varying the intensity of the incident light beam. Under these conditions, the oxidation of the enzyme by excess O_2 is rate limited by NO dissociation from reduced cytochrome a_3. The observed rate constant is plotted as a function of relative intensity of the incident light beam, expressed as counts detected at 550 nm. The intercept is the intrinsic dissociation rate constant, $k_{off} = 3.5 \times 10^{-3}\ s^{-1}$, in the dark.

RESULTS

We previously reported (3) the rate constant for the dissociation of NO from fully reduced beef heart cytochrome *c* oxidase in detergent solution, measured using two different methods to scavenge rapidly free NO and thus assess the rate of the reaction:

$$[\text{Cyt } a_3^{2+}\text{-NO Cu}_B^{+}] \xrightarrow{k_{off}} [\text{Cyt } a_3^{2+}\text{Cu}_B^{+}] + \text{NO}$$

We subsequently discovered that the value of $k_{off} = 0.1\ s^{-1}$ is not the intrinsic rate constant because of a marked increase in the dissociation rate due to the photochemical effect of the bright observation white light, as may have been anticipated based on the results of Boelens *et al.* (17). This effect prompted us to investigate more closely the photochemistry of the process. The apparent rate constant of NO dissociation increases linearly with light intensity (Fig. 1), and the value extrapolated at zero light ranges from 1 to $5 \times 10^{-3}\ s^{-1}$. This rate constant (k_{off}) has been further substantiated using a different experimental setup, and from the results the best estimate is $k_{off} = 3.9 \times 10^{-3}\ s^{-1}$ at 20°C, and $1.2 \times 10^{-2}\ s^{-1}$ at 37°C, with an activation energy $E^* = 7.2$ kcal mol^{-1}, as calculated from the temperature dependence in the range 9 to 37°C.

The light sensitivity of the cyt a_3^{2+}-NO adduct was exploited to prove the mechanism of inhibition of respiration by NO. As shown in Fig. 2, addition of an excess of NO stops O_2 consumption, but eventually respiration starts again if an excess of Hb-O_2 is added to scavenge free NO. When these experiments were carried out either in the dark or under illumination with white light, a surprising result was observed, in that the effect of light on the oxygraphic profile depends on cytochrome *c* concentration (Fig. 2). At the higher cytochrome *c* concentrations ($\geq$10 μM), O_2 consumption in the dark recovered quite slowly (several minutes) after addition of HbO_2, the time course being autocatalytic and compatible with the rate of NO dissociation from reduced cyt a_3^{2+}. If the reaction chamber was illuminated with a bright light however, immediate recovery of respiration was observed. When the assay was carried out in the lower cytochrome *c* concentration regime ($\leq$1 μM), recovery of respiration was faster and essentially simultaneous with addition of HbO_2 even in the dark; moreover and possibly more significant we observed that light has no effect. As seen in Fig. 2, the rate of O_2 consumption in the dark, calculated at a fixed time ($\sim$60 s) after scavenging free NO with HbO_2, normalized for the initial (fully active) rate, approaches maximal value at the lower cytochrome *c* concentrations, and tends to $\sim$0.3 at the higher cytochrome *c*, where recovery in the dark is slow but illumination has a big effect. These experiments show that when the reductive pressure is high, the NO-inhibited species is cyt a_3^{2+}-NO as proven by the effect of illumination which increases the NO dissociation rate. On the other hand when the reductive pressure is low photosensitivity is lost, presumably because the prevailing inhibited species is cyt a_3^{3+}-NO_2^-, which (like all adducts of ferric hemeproteins (18)) is not photosensitive.

In order to assess the oxidation and ligation state of the enzyme, particularly of the binuclear site, during the different phases of turnover in the presence of NO and at different concentrations of reductants, we have carried out stopped flow experiments replacing cytochrome *c* with ruthenium hexamine (19). As shown in

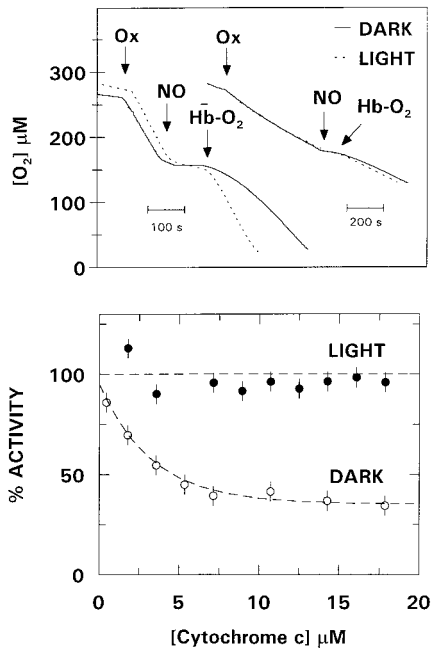


FIG. 2. Effect of light on respiration rate and NO-inhibition. Top: time courses of O_2 consumption catalyzed by cytochrome oxidase (50 nM) in the presence of 10 mM ascorbate, 200 μM TMPD and horse heart cytochrome *c* either 18 μM (left) or 1.8 μM (right). Experiments were carried out in the dark (solid traces) or under illumination (dotted traces, see Material and Methods for details). The rate of O_2 consumption catalyzed by addition of the enzyme (OX), drops dramatically after addition of 1.2 μM NO (NO); reversal of NO inhibition was initiated by addition of 4 μM oxy hemoglobin (HbO_2) to scavenge rapidly all free NO. At higher cytochrome *c* concentration (left), recovery of respiration starts very slow in the dark, but picks up under illumination, consistently with the rate of NO dissociation from reduced cytochrome a_3. At lower cytochrome *c* concentration (right), reversal of NO inhibition and recovery of initial respiration rate is quicker and independent of illumination. Bottom: the experiment reported above was repeated at different cytochrome *c* concentrations from 0.4 to 18 μM. Oxidase activity, estimated by fitting the best tangent to each time course at $t \approx 60$ s after HbO_2 addition, and normalized for the activity displayed before NO addition (100%), is plotted as a function of [cytochrome *c*]. Under illumination, about 60 s after HbO_2 addition the enzyme had already recovered completely its control activity. In the dark a different behaviour was observed depending on the reductive pressure; namely (i) at the highest [cytochrome *c*], recovery of activity is slow and thus the fraction of reactivated enzyme $\approx$ 60 s after HbO_2 addition is only $\approx$30% (a value consistent with the intrinsic dissociation rate constant of NO from reduced cytochrome a_3 at 20°C, see text); (ii) as the cytochrome *c* concentration is lowered enzyme reactivation after NO removal is much faster, pointing to a different inhibition pattern.

Fig. 3, the overall time course shows that in the absence of NO the enzyme populates the half reduced state approaching a steady state and eventually becomes fully reduced (on O_2 exhaustion). In the presence of NO, on the other hand, two different time patterns were observed, depending on ruthenium hexamine concentration. At high reductant (250 μM), the reduced cyt a_3^{2+}-NO is the inhibited species, which forms within $\sim$1 s and in the presence of O_2 recovers activity. However, at low ruthenium hexamine (5 μM) the oxidized enzyme initially approaches a steady state, but thereafter changes to a different combination of intermediates including a significant fraction of the inhibited NO_2^--bound adduct. As detailed in Fig. 3, this species has the spectral features of the cyt a_3^{3+}-NO_2^- bound enzyme, and inhibition is associated to accumulation of reduced cyt *a*. In the presence of excess reductants, the nitrite-inhibited enzyme decays eventually into the fully reduced species (unpublished data).

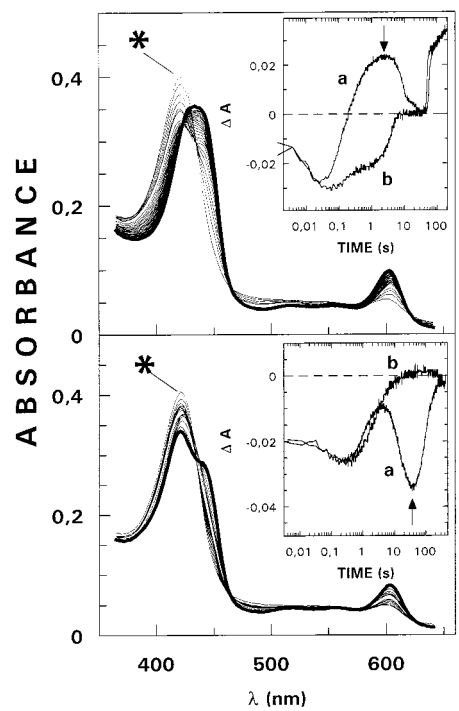


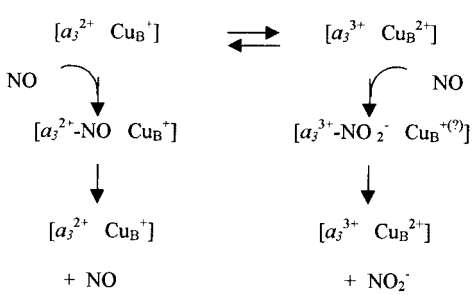
FIG. 3. Optical investigation of NO-inhibited oxidase at high and low reductive pressure. Oxidized cytochrome *c* oxidase (4 μM) was degassed and anaerobically mixed in the diode-array stopped-flow with a solution containing 10 mM ascorbate and ruthenium hexamine either 500 (top) or 10 μM (bottom), in both cases with or without 40 μM NO. Top: absorption spectra corresponding to time course **a** in the inset, displayed in sequence from 2.5 ms to 3 s (indicated by the arrow). Ruthenium hexamine = 500 μM and NO = 40 μM (concentrations before mixing). First spectrum is the starting oxidized enzyme (asterisk); last spectrum (thick) is the fully-reduced nitrosylated enzyme. Bottom: absorption spectra corresponding to time course **a** in the inset, displayed from 2.5 ms to 50 s (indicated by the arrow). Ruthenium hexamine = 10 μM and NO = 40 μM (concentrations before mixing). First spectrum is the oxidized enzyme (asterisk); last spectrum (thick spectrum) is mainly representative of nitrite-inhibited oxidase. Insets: time courses observed at 434–464 nm with NO (**a**) or without NO (**b**). Baseline corresponds to the absorption level of the steady-state spectrum, in the absence of NO (**b**), where the enzyme approaches a steady-state and eventually becomes fully reduced, when O_2 is exhausted (at the longer times, not shown). In the presence of NO (**a**), the enzyme approaches an inhibited state (shown by the arrow), whose optical spectrum feature is remarkably different depending on the reductive pressure (cfr. spectra in top and bottom panels). Activity is recovered in both cases because trace **a** in both insets approaches eventually the steady-state levels observed in the absence of NO.

DISCUSSION

NO inhibits rapidly and reversibly mitochondrial respiration (4, 5, 20), by reacting with the binuclear site of complex IV (1, 3, 21). Inhibition depends on the relative concentration of O_2 and NO (3, 22, 23), which implies that the reduced binuclear centre of cytochrome *c* oxidase is the target; the inhibited state seemed to correspond to the cyt a_3^{2+}-NO state (1, 3). Under turnover conditions and relatively low NO concentrations, the nitrosylated species recovers spectral features and activity at a speed consistent with the rate of NO dissociation from the site. We have now direct evidence that the rate constant of $k'_{off} = 0.1\ s^{-1}$ reported by us (3) was largely overestimated since the experiments were carried out under a bright illumination. Because of the photosensitivity of the cyt a_3^{2+}-NO adduct (17), the dissociation rate constant increases with light intensity (Fig. 1), and at zero light, the correct value is $k_{off} = 3.5 \times 10^{-3}\ s^{-1}$ at 20°C (and $k_{off} = 1 \times 10^{-2}\ s^{-1}$ at 37°C). We have taken advantage of this unique feature to clarify different mechanisms of inhibition by NO.

In 1998, it was shown by Torres *et al.* (21) and confirmed by us (13), that the reaction of NO with the (chloride-free) oxidised binuclear site involves oxidation to NO^+ and reduction of cyt *a* (and Cu_A), presumably *via* Cu_B. As confirmed in this paper, in the absence of reductants the nitrite formed in the active site binds to cyt a_3^{3+} (13, 21) and the enzyme is inhibited. Intramolecular reduction of the binuclear site promptly reverts inhibition without detection of cyt a_3^{2+}-NO; the time of recovery (seconds) depends on the concentration of reductants and in turn, on the number of turnovers elapsed (unpublished observations). Thus, NO_2^- bound to ferric cyt a_3^{3+} dissociates from the binuclear site without being re-reduced to NO but probably as the acid (similarly to other anions such as CN^-, N_3^- and Cl^-) (24). Therefore, accumulation of the nitrite-bound species reveals an important and somewhat peculiar function of cytochrome c oxidase, namely the oxidative degradation of NO to harmless NO_2^- (25).

In summary, two different mechanisms of inhibition of cytochrome c oxidase by NO, involving alternative complexes with cyt a_3 and different reaction products, have been substantiated, namely:



In this paper we show that, under turnover conditions, both mechanisms may be operational depending on the reductants "pressure", based on the fact that the cyt a_3^{2+}-NO is light-sensitive, while cyt a_3^{3+}-NO_2^- is not; the oxidation state of Cu_B in the $[a_3^{3+}\text{-}NO_2^- Cu_B^{+(?)}]$ adduct, is not uniquely assigned and should be assessed. The light sensitivity of cyt a_3^{2+}-NO has been exploited to probe its accumulation during turnover at different concentrations of reductant (cytochrome *c* or ruthenium hexamine). In the high concentration regime, cyt a_3^{2+}-NO is the prevailing inhibited state, as indicated spectroscopically and proven by the effect of light on O_2 consumption rate (see Fig. 2). On the other hand at lower reductants, the inhibited species is cyt a_3^{3+}-NO_2^-, which is light insensitive like all complexes of ferric hemeproteins (18), and has specific spectroscopic features (Fig. 3). As a matter of fact, inhibition is unequivocally demonstrated by the accumulation of reduced cytochrome *a*, when the nitrite-bound enzyme is accumulated.

On the basis of these results, we conclude that *in vitro* the mechanism by which cytochrome *c* oxidase is inhibited by NO is twofold depending on the reductant concentration, i.e.: either *via* nitrosylation of cyt a_3^{2+}, or *via* reaction of NO with the oxidised binuclear centre and accumulation of the (nitrite-bound) cyt a_3^{3+} NO_2^- species. The inhibition mechanism that prevails under turnover conditions will depend on several parameters, including the relative occupancy of the intermediates in the O_2 cycle and their intrinsic reactivity with NO (which ranges from 10^8 to $10^4\ M^{-1}\ s^{-1}$ (11, 13). Here we have shown that it is possible, by simply illuminating the respiring system, to reveal which of the two mechanisms is active. It should be emphasized that, oxidation of NO to nitrite and its relatively fast release (into the bulk!) accounts for catalytic NO degration and ultimately cell detoxification (21, 25).

Finally, the intrinsic dissociation rate constant of cyt a_3^{2+}-NO ($k_{off} = 10^{-2}\ s^{-1}$ at 37°C) is compatible with the rate of mitochondrial respiration recovery measured in cells and tissues (27, and ref. therein), the former inhibited by adding to the medium an excess of NO-donor (NOR_1) (26). This finding and the likelyhood that at higher reductant concentration binding of NO to reduced cyt a_3^{2+} prevails may suggest that *in vivo*, and under similar conditions, the inhibition of cytochrome *c* oxidase by NO is mainly exerted *via* formation of the cyt a_3^{2+}-NO adduct; this is supported by the well documented competition between O_2 and NO (3, 22, 23). On the other hand, the inhibition mechanism based on formation of the cyt a_3^{3+}-NO_2^- and observed at lower reductant concentration would prevail under pathophysiological conditions, such as the upstream inhibition of the respiratory chain or mitochondrial swelling, leading to critical decrease of reduced cytochrome *c* at the cytochrome oxidase binding site.

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